Supplementary Experimental Procedures

siRNAs

siRNA *SMART*pools (Dharmacon) contain four different siRNA oligonucleotides that target the same gene. Single siRNAs correspond to one of the four siRNAs in the relevant *SMART*pool.

Supplementary Table 1. siRNA *SMART*pools and duplexes

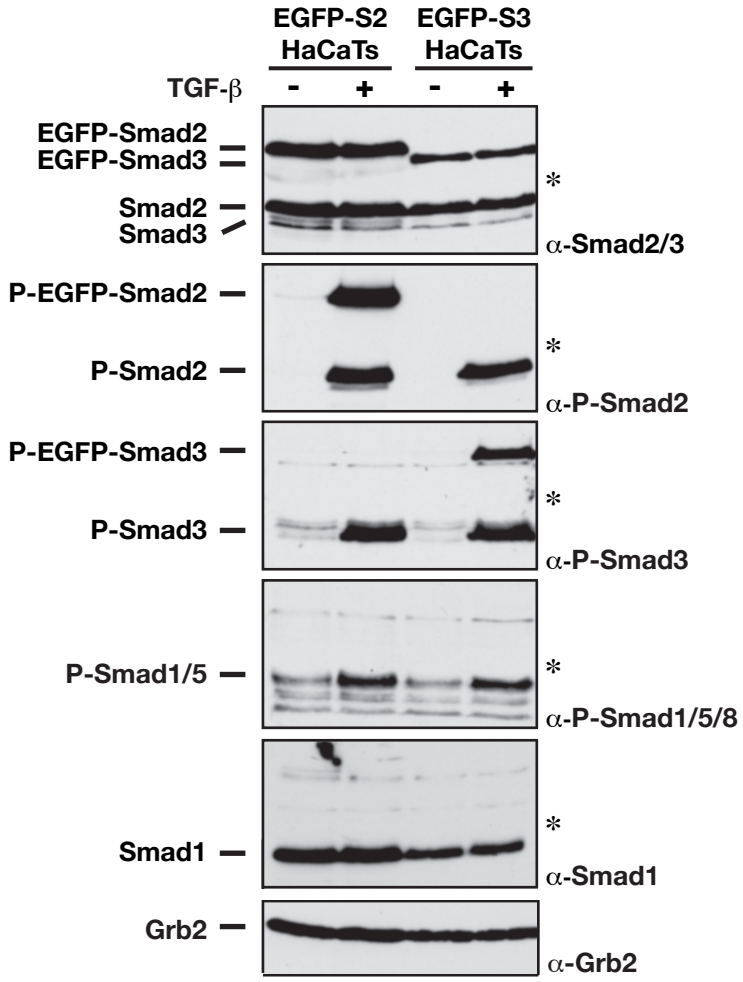
SMARTpool siRNA or siRNA duplex	***Catalogue number*** Mouse siRNA	***Catalogue number*** Human siRNA
Smad1	M-055762-00 or D-0055762-01* or D-0055762-03*	M-012723-00
Smad2	M-040707-00 or D-040707-03*	M-003561-00
Smad3	M-040706-00	M-020067-00
Smad5	M-057015-00	M-015791-00
Smad8	M-046344-00	-
ALK1	M-043004-00	M-005302-02
ALK2	D-042047-03* D-042047-04*	M-004924-01
ALK3	D-040598-04*	M-004933-03
ALK5	D-040617-02*	M-003929-01
ALK6	M-051071-00	M-004934-01
TβRII	M-040618-00	M-003930-01
BMPRII	L-040599-00	-

* indicates siRNA duplexes used.

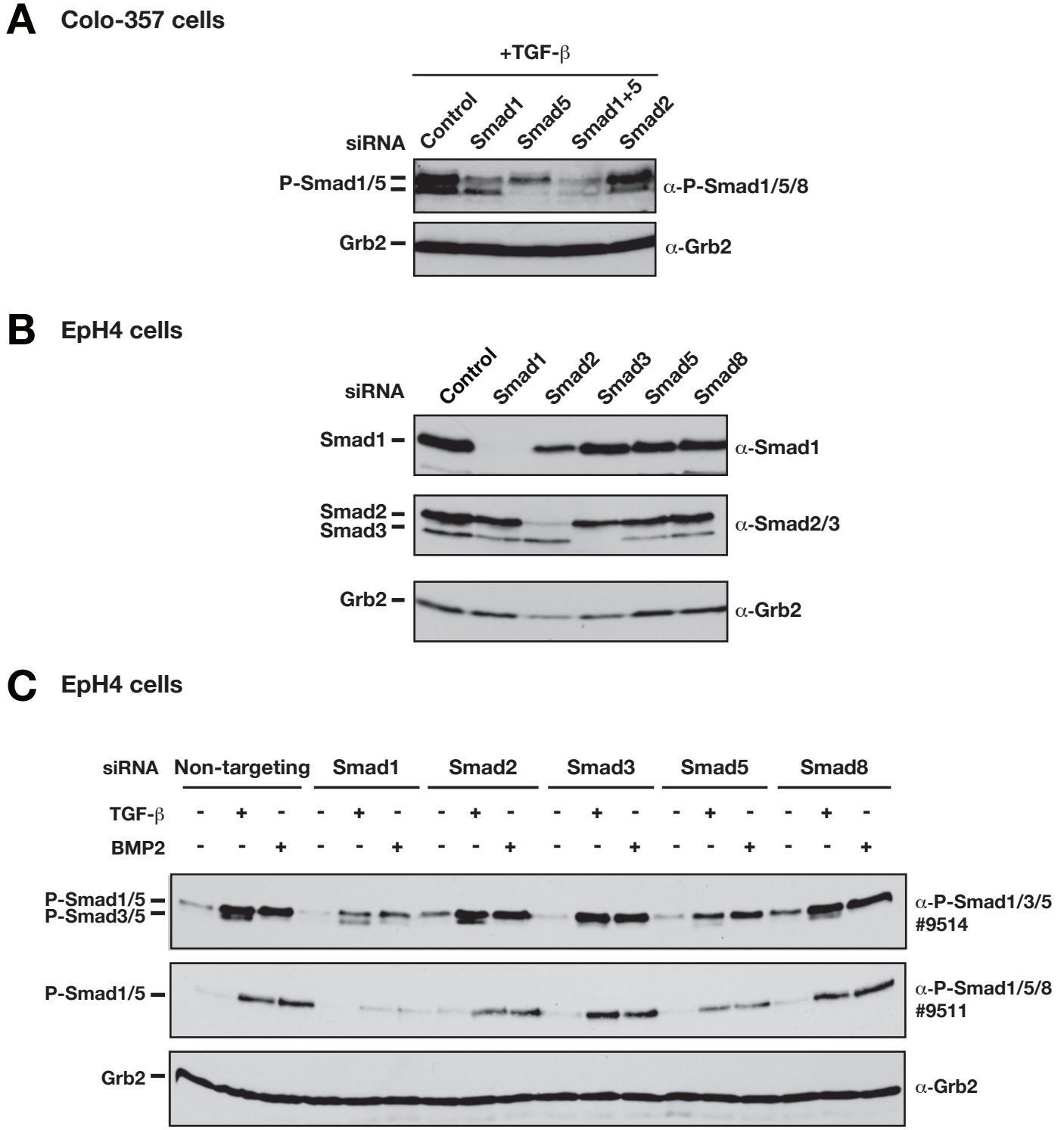
Control siRNAs used were RISC-Free siRNA (catalogue number D-001220-01-05) or non-targeting (catalogue number D-001206-13-05).

Supplementary Table 2. Oligonuclotides used in RT-PCR

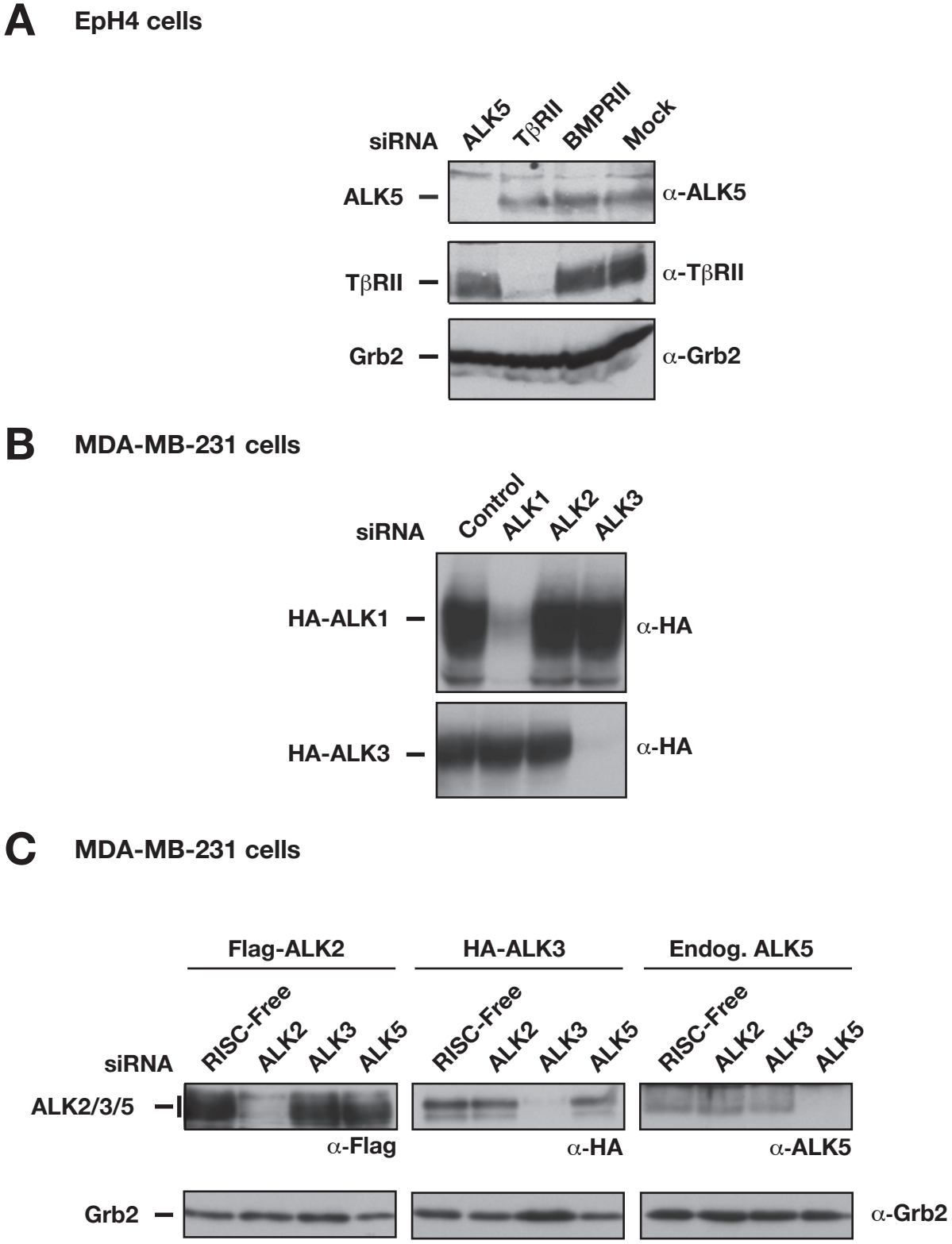
Name	Human Oligonucleotide Sequence	Mouse Oligonucleotide Sequence
ALK1-Fwd	CCTTGCTGGCCCTGGTGGCCCT	GCAGTGTTGCATTGCAGACC
ALK1-Rev	GTGGGCAATGGCTGGTTTG	GCACTCTCTCATCATCTGG
ALK2-Fwd	GAAGGGCTCATCACCACCAAT	GACAGCACTCTAGCGGAACTAC
ALK2-Rev	GAACGGTGGCTTGTAATCCTC	GACTGCCAGGCCCAAATCTGC
ALK3-Fwd	GCACATTGCTTTGCCATCATA	GCCACCTCCACACAGAAATT
ALK3-Rev	CATTTGCCCATCCATACTTCT	TTACATCCTGGGATTCAACC
ALK4-Fwd	GAGATCGTGGGCACCCAAGGG	CTCCTCCTTCTTCCCCCTTG
ALK4-Rev	AGCTGGGAGAGGGTCTTCTTG	CTCCATGTCCAACCTCTGGC
ALK5-Fwd	GATGGGCTCTGCTTTGTCTCT	CCTTTCATTTCAGAGGGCAC
ALK5-Rev	TGTCTTTATTGTCTGCTGCTA	CCACTTGCTGTGGACAGAGC
ALK6-Fwd	ACACCACAGGGCTTTACTTAT	CACCAAGCGCTATATGCCTC
ALK6-Rev	AATTGCTGGTTTGCCTTGAGT	CTCTCTTCCAGGAAAGTCTG
ALK7-Fwd	GCAACAACATAACACTGCACCTT’	CATCTATTCGGTGGGGCTGG
ALK7-Rev	CAATTGTCCTTTGAACCAACAGA	CGGGAAGGAAAGCTGTGAGC
GAPDH-Fwd	ACCACAGTCCATGCCATCAC	
GAPDH-Rev	TCCACCACCCTGTTGCTGTA	
Grb2-Fwd		GATCAACATCCGTGTCCAGG
Grb2-Rev		AACATCATGCACTGGACAGG



Supplementary Figure 1. The P-Smad1/5/8 antibody is specific for Smad1/5/8 and does not recognize the phosphorylated form of Smad2 or Smad3.
HaCaT cells stably expressing EGFP-Smad2 (EGFP-S2) or EGFP-Smad3 (EGFP-S3) (Nicolás et al., 2004 J. Cell Sci. 117, 4113-4125) were treated with or without TGF-β1 for 1 hour. Whole cell extracts were analyzed by Western blotting using antibodies against Smad2/3, P-Smad2, P-Smad3, P-Smad1/5/8, Smad1, and Grb2 as a loading control. P-Smad2 and P-Smad3 recognize endogenous and EGFP-Smad2 and EGFP-Smad3, respectively, whereas P-Smad1/5/8 only recognizes endogenous P-Smad1/5. The asterisk corresponds to the position of a 66.5 kD protein marker.

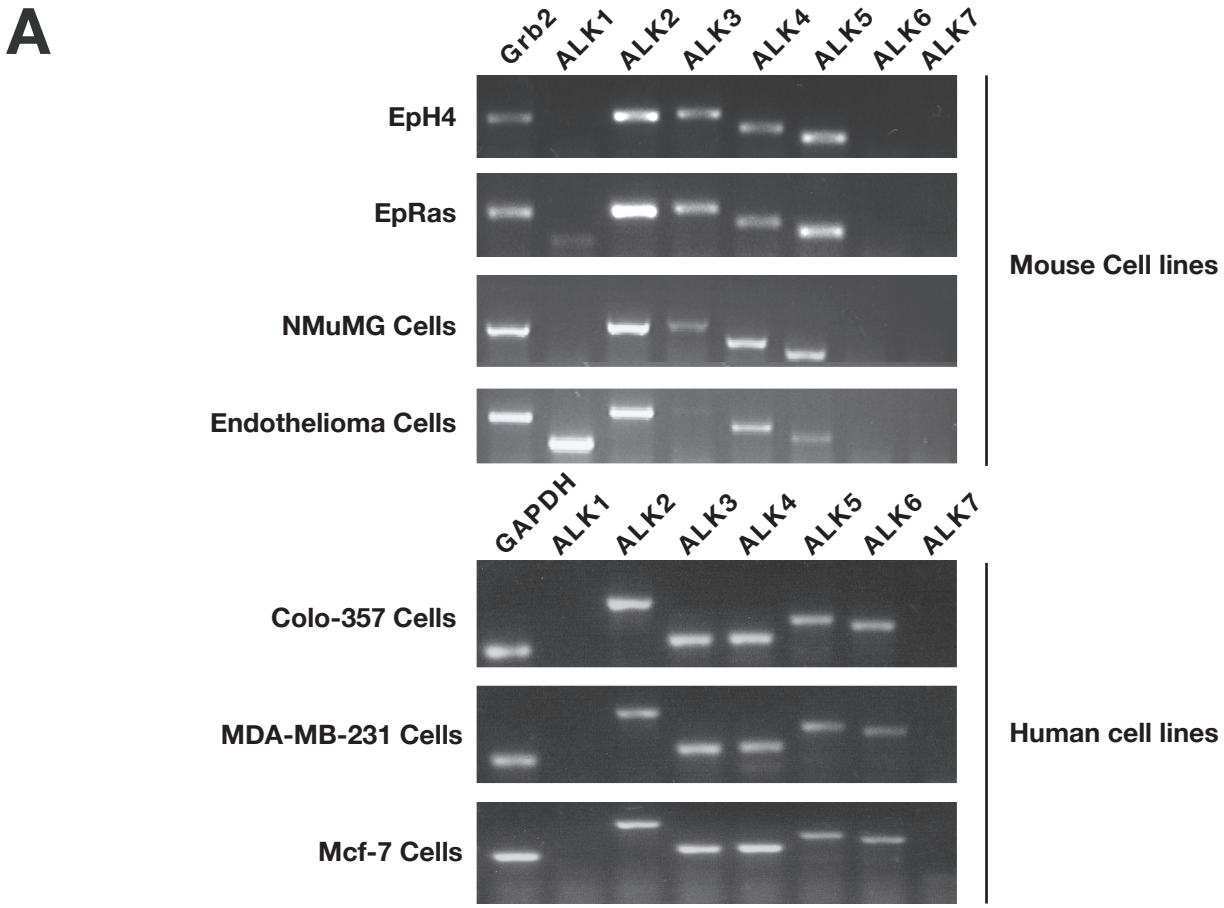


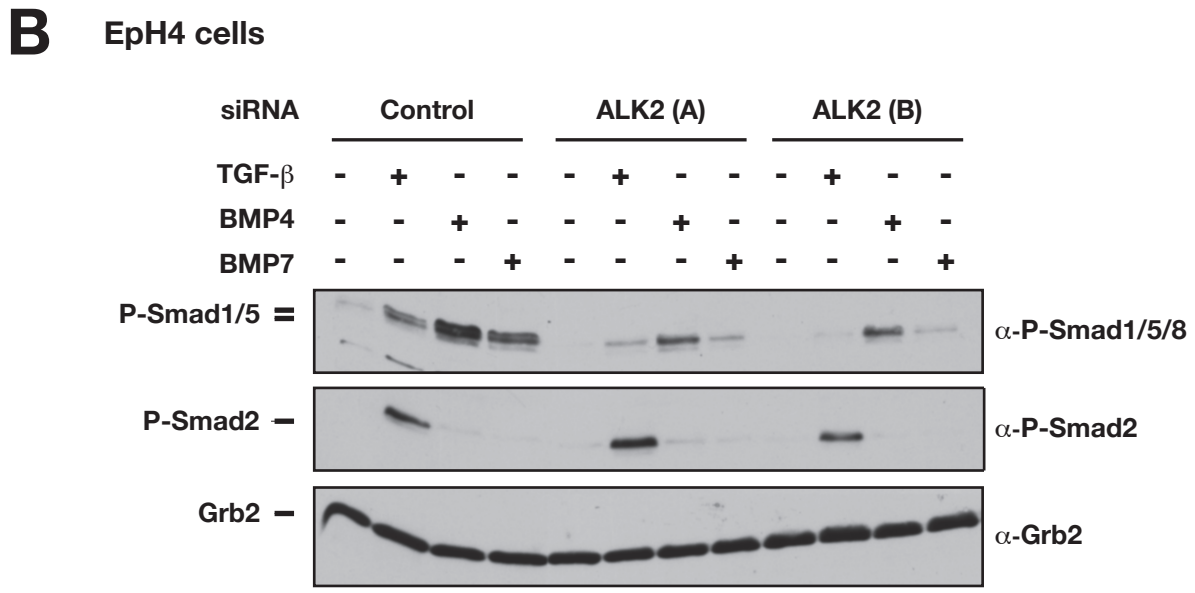
Supplementary Figure 2. Smad1 and Smad5 are phosphorylated in response to TGF-β
A. Colo-357 cells were transfected with siRNA *SMART*pools against Smad1, Smad2, Smad5 or a control siRNA oligo, as indicated. After 72 hours cells were treated with TGF-β1 for 45 min, then whole cell extracts were analyzed by Western blotting using antibodies against P-Smad1/5/8 and Grb2. The upper band is comprised of a mixture of Smad1 and Smad5, whereas the lower band is primarily Smad5. **B.** EpH4 cells were transfected with siRNA *SMART*pools against the individual R-Smads, or a control siRNA oligo, as indicated. After incubation for 72 hours, cells were analyzed by Western blotting using antibodies against Smad1, Smad2/3 and Grb2. Note that the lane containing the sample transfected with Smad2 siRNA *SMART*pool is slightly under loaded. **C.** Specificity of the phospho-Smad1/3/5 antibody. The samples that were Western blotted using the anti-P-Smad1/5/8 antibody (Figure 1C) were Western blotted using the anti-P-Smad1/3/5 antibody (upper panel). The siRNA knockdowns indicate that the top band recognized by this antibody is a mixture of phosphorylated Smad1 and Smad5 and the bottom band is predominantly phosphorylated Smad3 with a small amount of phosphorylated Smad5. The anti P-Smad1/5/8 blot and the anti Grb2 blot from Figure 1C are shown again here for comparison.



Supplementary Figure 3. Efficiency and specificity of receptor knockdown

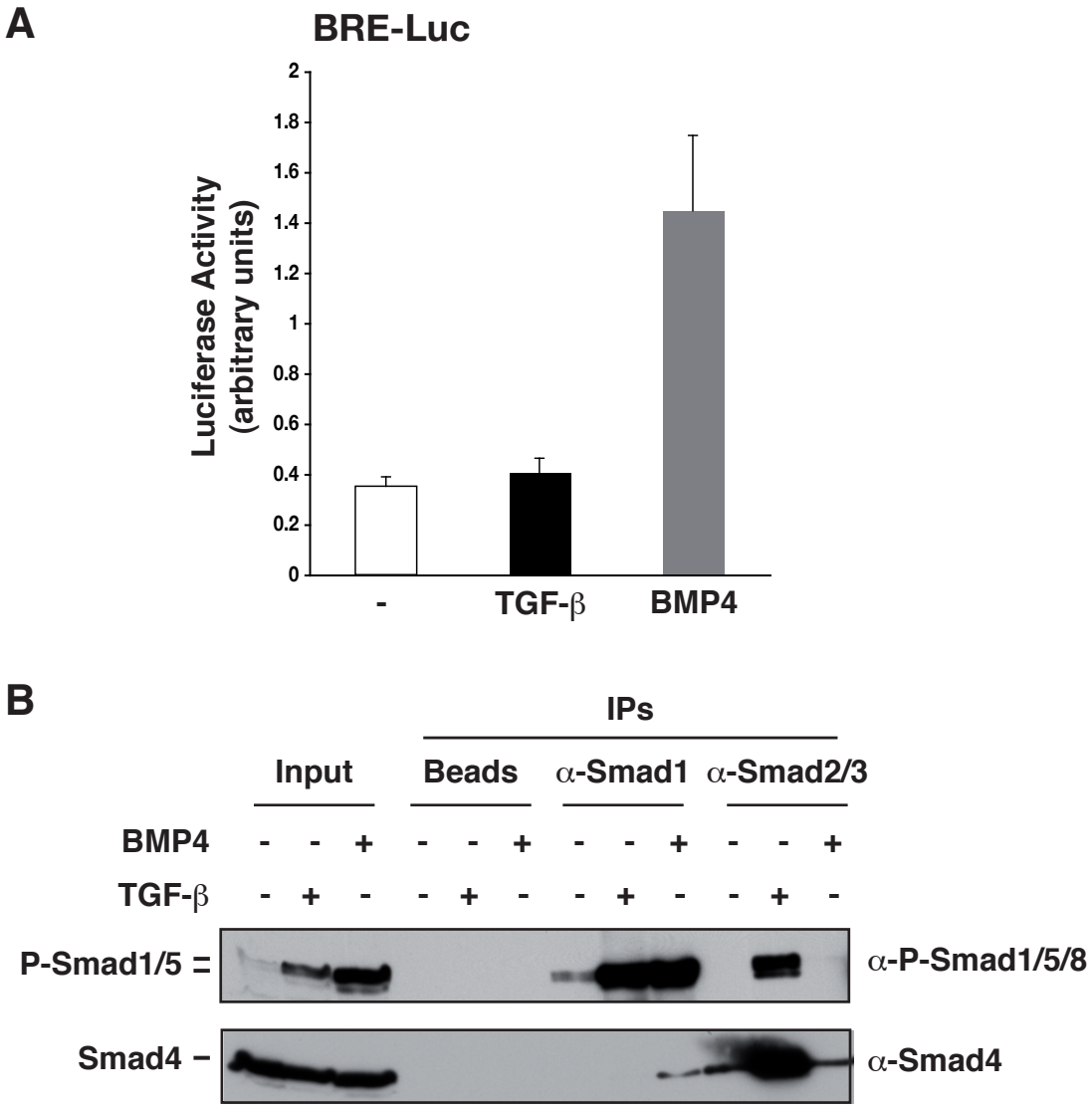
A. Efficient knockdown of ALK5 and TβRII by siRNA silencing in EpH4 cells. EpH4 cells were transfected with siRNA SMARTpools against TβRII, BMPRII, an ALK5 siRNA duplex or were mock transfected. Whole cell extracts were treated with 50 units of PNGaseF for 1 hour at 37 °C to remove N-linked glycosyl groups ((Dorey and Hill, 2006 Dev. Biol. 292, 303-316)) and then were analyzed by Western blotting using antibodies against ALK5, TβRII and Grb2. **B.** MDA-MB-231 cells were transfected with siRNA *SMART*pools against ALK1, ALK2, ALK3 or a control siRNA oligo. After 24 hours incubation, cells were transfected with plasmids expressing either HA-ALK1 or HA-ALK3. Whole cell extracts were analyzed by Western blotting using HA-HRP. **C.** MDA-MB-231 cells were transfected with siRNA *SMART*pools against ALK2, ALK3, ALK5 or a control siRNA oligo. After 48 hours incubation, cells were transfected with plasmids expressing either FLAG-ALK2 or HA-ALK3 or were untransfected. Whole cell extracts were analyzed by Western blotting using HA-HRP, anti-FLAG-HRP, anti-ALK5, or anti-Grb2 as a loading control.





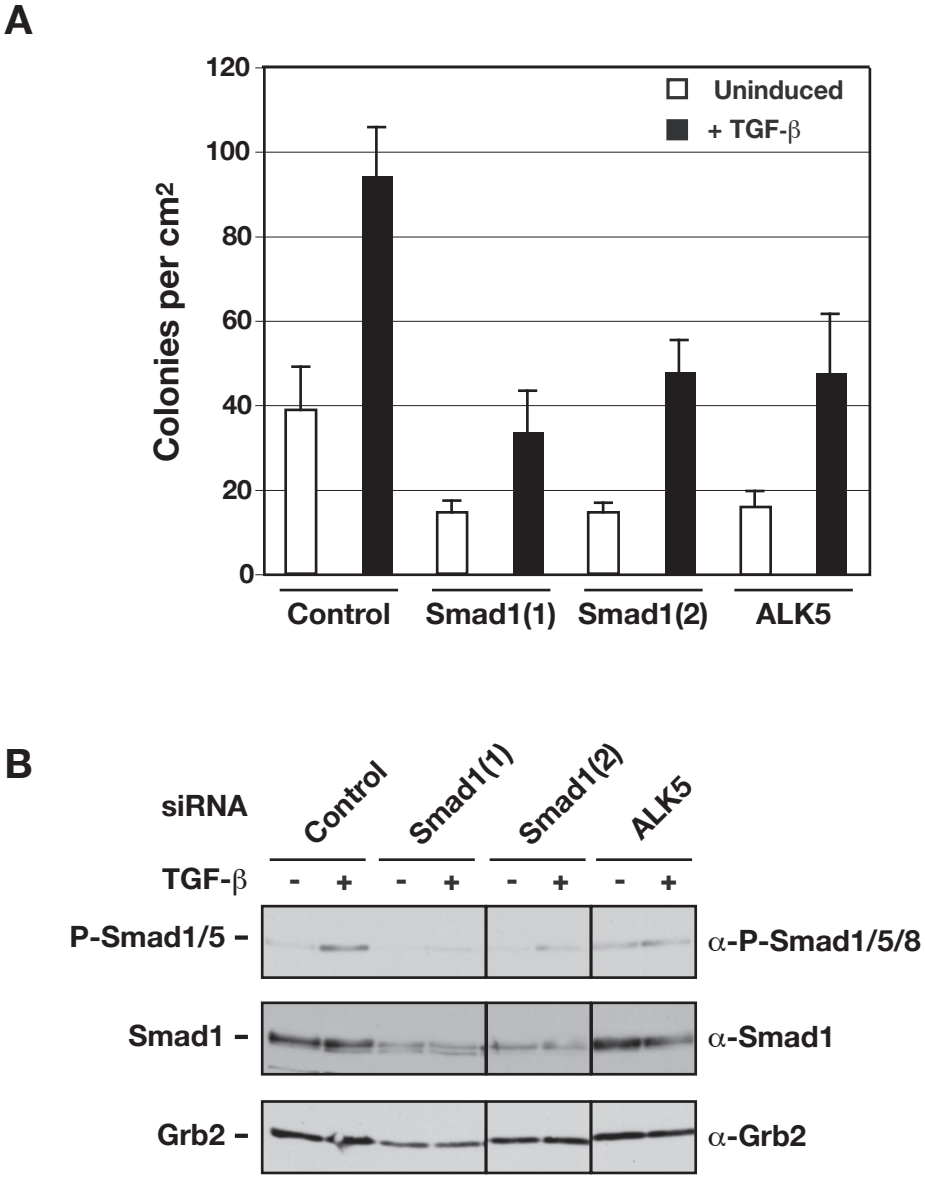
Supplementary Figure 4. ALK2 and ALK3 are required for Smad1/5 phosphorylation in response to TGF-β

A. Expression of TGF-β type I receptors. The expression of genes encoding the ALK receptors was analyzed by RT-PCR. RNA was isolated from the cell lines indicated and RT-PCR was performed using primers specific for *ALKs 1–7*, and *Grb2* or *GAPDH* as a control. The amplified product is indicated at the top of the panel. Expression of *ALKs 2–5* is observed in all cell lines. Mouse endothelioma cells (Rohnelt et al., 1997 Int. Immunol. 9, 435-450) were used as a positive control for *ALK1* mRNA expression. **B.** EpH4 cells were transfected with two individual siRNA duplexes against ALK2 or a control siRNA oligo. Cells were then either uninduced or stimulated with TGF-β1, BMP4 or BMP7 for 45 min, as indicated. Whole cell extracts were analyzed by Western blotting using antibodies against P-Smad1/5/8, P-Smad2 and Grb2 as a loading control.



Supplementary Figure 5. TGF-β-induced phospho-Smad1/5 fails to activate transcription from a BMP-responsive element in NMuMG cells because it forms mixed R-Smad complexes with Smad2/3.

A. Luciferase reporter assays in NMuMG cells. Cells were transfected with BRE-Luc and induced with TGF-β1 or BMP4 for 8 hrs as indicated. Luciferase activity was assayed and normalized. The data are the means and standard deviations of three independent experiments. **B.** Interaction of Smad1/5 with Smad2/3 was assayed by immunoprecipitation with anti-Smad antibodies followed by Western blotting. NMuMG cells were either untreated or stimulated with either TGF-β1 (2 ng/ml) or BMP4 (20 ng/ml) for 45 min before lysis. Whole cell extracts were prepared and equal amounts of protein were immunoprecipitated with antibodies against Smad1, Smad2/3 or with beads alone. The immunoprecipitation reactions (IPs) were analyzed by Western blotting with antibodies against Smad4 and phospho-Smad1/5/8 (P-Smad1/5/8). As a control, inputs are also shown on the left of the panel.



Supplementary Figure 6. Smad1 is required for anchorage-independent growth in soft agar.
A. Activation of Smad1 in combination with Smad2/3 by TGF-β is required for the growth of EpRas cells in soft agar in response to TGF-β. EpRas cells were transfected with siRNA duplexes against Smad1, ALK5 or a control siRNA oligo, as indicated. After 48 hours, the cells were assayed for their ability to grow in soft agar in the absence or presence of 2 ng/ml TGF-β as described in Materials and Methods. After 12 days, the number of colonies was assessed by staining with MTT. The mean and SD of three replicate wells of a representative experiment is shown. **B.** Confirmation of knockdown of Smad1 and loss of TGF-β-induced phosphorylation of Smad1/5 by Western blot analysis.